

20011110/018113

JC09 Rec'd PCT/PTO 14 DEC 2001

Practitioner's Docket No. 806.01-US1

CHAPTER II

**TRANSMITTAL LETTER
TO THE UNITED STATES ELECTED OFFICE (EO/US)
(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)**

PCT/DE00/01950	15 June 2000 (15.06.00)	16 June 1999 (16.06.99)
International Application Number	International Filing Date	International Earliest Priority Date

TITLE OF INVENTION: Method of Modifying Peptide Synthetases Such That They Can N-Methylate Their Substrate Amino Acids

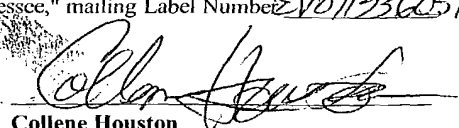
APPLICANT(S): ActinoDrug Pharmaceuticals GmbH; Keller, Ullrich; Schauwecker, Florian

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: EO/US

1. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. Section 371:
 - a. This express request to immediately begin national examination procedures (35 U.S.C. Section 371(f)).
 - b. The U.S. National Fee (35 U.S.C. Section 371(c)(1)) and other fees (37 C.F.R. Section 1.492) as indicated below:

CERTIFICATION UNDER 37 C.F.R. SECTION 1.10*

I hereby certify that this paper, along with any document referred to, is being deposited with the United States Postal Service on this date 12/13/01, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number E1011336259 US addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231


Collene Houston

CLAIMS FEE*	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
BASIC FEE	TOTAL CLAIMS	15 - 20 =	0	x \$18.00 =	\$0.00
	INDEPENDENT CLAIMS	1 - 3 =	0	x \$84.00 =	\$0.00
	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$280.00				\$0.00
	U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in Section 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in Section 1.445(a)(2) to the U.S. PTO: has not been paid (37 C.F.R. Section 1.492(a)(3))\$1,040.00				\$1,040.00
	Total of above Calculations				= \$1,040.00
SMALL ENTITY	Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed. (note 37 CFR Sections 1.9, 1.27, 1.28)				- \$520.00
	Subtotal				\$520.00
	Total National Fee				\$520.00
	Fee for recording the enclosed assignment document \$40.00 (37 C.F.R. Section 1.21(h)). See attached "ASSIGNMENT COVER SHEET".				\$0.00
TOTAL	Total Fees enclosed				\$520.00

3. A copy of the International application as filed (35 U.S.C. Section 371(c)(2)) has been transmitted by the International Bureau.

4. A translation of the International application into the English language (35 U.S.C. Section 371(c)(2)) is transmitted herewith.

5. Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. Section 371(c)(3)) have not been transmitted. Applicant chose not to make amendments under PCT Article 19.

6. A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. Section 371(c)(3)) has not been transmitted for reasons indicated in section 5.

7. A copy of the international examination report (PCT/IPEA/409) is transmitted herewith.

8. Annex(es) to the international preliminary examination report is/are transmitted herewith.
9. An oath or declaration of the inventor (35 U.S.C. Section 371(c)(4)) complying with 35 U.S.C. Section 115 will follow.
10. An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a) is transmitted herewith.
11. An Information Disclosure Statement under 37 C.F.R. Sections 1.97 and 1.98 will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. Section 371(c).
12. Additional documents:
 - a. Copy of request (PCT/RO/101)
 - b. International Publication No. WO00/77220 with Specification, claims and drawing
 - c. Preliminary Amendment deleting multiple dependencies
13. The above items are being transmitted before 30 months from any claimed priority date.

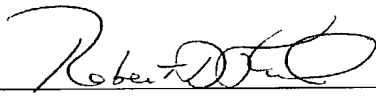
AUTHORIZATION TO CHARGE ADDITIONAL FEES

The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No.: 500341

37 C.F.R. Section 1.492(a)(1), (2), (3), and (4) (filing fees)
37 C.F.R. Section 1.492(b), (c), and (d) (presentation of extra claims)
37 C.F.R. Section 1.17 (application processing fees)
37 C.F.R. Section 1.17(a)(1)-(5) (extension fees pursuant to Section 1.136(a))
37 C.F.R. Section 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 20 months after the priority date).

Date: 14 Dec 2001

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Rec'd PCT/PTO

29 APR 2002

#5

Practitioner's Docket No. 806.01-US1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: ActinoDrug Pharmaceuticals GmbH; Keller, Ullrich; Schauwecker, Florian

Application No.: 10/018,113

Group No.: To be determined

Filed: 12/14/01

Examiner: To be determined

For: Method of Modifying Peptide Synthetases such That They can N-Methylate their Substrates

Box PCT

Assistant Commissioner for Patents

Washington, D.C. 20231

Attention: EO/US

**SUBMISSION OF "SEQUENCE LISTING," COMPUTER READABLE COPY,
AND/OR AMENDMENT PERTAINING THERETO
FOR BIOTECHNOLOGY INVENTION CONTAINING NUCLEOTIDE
AND/OR AMINO ACID SEQUENCE**

1. This replies to the Office Letter DATED March 19, 2002.

A copy of the Office Letter is enclosed.

IDENTIFICATION OF PERSON MAKING STATEMENT

2. I, Martin Fessenmaier, state the following:

ITEMS BEING SUBMITTED

3. Submitted herewith is/are:

A copy of each "Sequence Listing" submitted for this application in computer readable form, in accordance with the requirements of 37 C.F.R. sections 1.821(e) and 1.824.

Because this submission is made in fulfilling the requirement under 37 C.F.R. section 1.821(g), a statement that the submission includes no new matter.

Also on the diskette is a copy of the sequence listing as originally filed in the corresponding PCT Application Number PCT/DE00/1950.

**STATEMENT THAT "SEQUENCE LISTING"
AND COMPUTER READABLE COPY ARE THE SAME
AND/OR THAT PAPERS SUBMITTED INCLUDES NO NEW MATTER**

4. I hereby state:

Each computer readable form submitted in this application, including those forms requested to be transferred from applicant's other application, is the same as the "Sequence Listing" to which it is indicated to relate.

All papers accompanying this submission, or for which a request for transfer from applicants' other application, introduce no new matter.

STATUS

5. Applicant is a small entity. A statement was already filed.

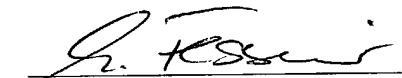
EXTENSION OF TERM

6. The proceedings herein are for a patent application and the provisions of 37 C.F.R. section 1.136 apply. Applicant believes that no extension of term is required. However, this conditional petition is being made to provide for the possibility that applicant has inadvertently overlooked the need for a petition for extension of time.

If any additional extension and/or fee is required, charge Account No. 500341.

SIGNATURE(s)

Date: 04/28/02



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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231

Inventor: **Ullrich Keller & Florian Schauwecker**

Serial No: **US national phase of PCT/DE00/01950**

Filed: **June 15, 2000**

For: **Method of Modifying Peptide Synthetases
Such That They Can N-Methylate Their
Substrate Amino Acids**

Examiner:

Art Unit:

PRELIMINARY AMENDMENT

The Honorable Commissioner
of Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

Please enter the following as a preliminary amendment.

IN THE CLAIMS

4. (Amended) Method according to claim 1, wherein the DNA fragment encoding for a domain with N-methyltransferase activity is cloned between the DNA regions encoding for the adenylation domain and for the ACP-domain of the PPS activation domain without N-methyltransferase activity by means of a single fusion site.
5. (Amended) Method according to claim 1, wherein the DNA fragment encoding for a domain with N-methyltransferase activity is cloned by means of two fusion sites.
6. (Amended) Method according to claim 4, wherein a DNA fragment encoding for a domain with N-methyltransferase activity is additionally encoding for an ACP-domain, an activation domain or a condensation domain.

7. (Amended) DNA, obtainable according to the method of claim 1.
9. (Amended) Method for the manufacture of a PPS with N-methyltransferase activity, wherein the DNA obtained according to the method of claim 2 encoding for a PPS with N-methyltransferase activity is expressed.
11. (Amended) PPS with N-methyltransferase activity, obtainable according to the method of claim 9.

REMARKS

The requested changes merely remove multiple dependencies.

Respectfully submitted,
Fish & Associates, LLP

Dated: 17 Dec 2001

By: 

Robert D. Fish
Reg. No. 33,880

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VERSIONS WITH MARKING TO SHOW CHANGES MADE**In the Claims**

4. (Amended) Method according to ~~any of~~ claims 1-3, wherein the DNA fragment encoding for a domain with N-methyltransferase activity is cloned between the DNA regions encoding for the adenylation domain and for the ACP-domain of the PPS activation domain without N-methyltransferase activity by means of a single fusion site.
5. (Amended) Method according to ~~any of~~ claims 1-3, wherein the DNA fragment encoding for a domain with N-methyltransferase activity is cloned by means of two fusion sites.
6. (Amended) Method according to ~~any of~~ claims 4-~~or~~ 5, wherein a DNA fragment encoding for a domain with N-methyltransferase activity is additionally encoding for an ACP-domain, an activation domain or a condensation domain.
7. (Amended) DNA, obtainable according to the method of ~~any of~~ claims 1-6,.
9. (Amended) Method for the manufacture of a PPS with N-methyltransferase activity, wherein the DNA obtained according to the method of ~~any of~~ claims 2-6, encoding for a PPS with N-methyltransferase activity is expressed.
11. (Amended) PPS with N-methyltransferase activity, obtainable according to the method of ~~any of~~ claims 9-10.

7/prts

JC13 Rec'd PCT/PTO 14 DEC 2001

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Method of modifying peptide synthetases such that they can N-methylate their substrate amino acids

Description

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The invention relates to the modification of peptide synthetases (PPS) such that they can N-methylate their substrate amino acids. This is achieved by a specific modification or replacement of the functional subunits
10 (activation domains) of these enzymes.

Peptide synthetases (PPS) are enzymes which synthesize peptides by a non-ribosomal mechanism. The peptides synthesized by the PPS (or derivatives thereof) are often of
15 pharmaceutical interest, e.g. penicillines, vancomycin, cephalosporin, pristinamycin or actinomycin D. The PPS have a modular set-up. Each module of a PPS recognizes, activates and binds one amino acid. Some PPS modules also accept unusual (non-proteinogenic) amino acids as substrates, e.g.
20 alpha-aminoadipinic acid (in penicillin) or phenylglycine (in pristinamycin). The synthesis of a peptide catalyzed by the PPS takes place by the enzyme-catalyzed condensation of the amino acids bound to the modules. This condensation is directed, namely in such a way that the substrate amino acid
25 bound to the first module of the PPS (referred to the N-terminus of the PPS) forms the start (N-terminus) of the synthesized peptide. Thus, the number and order of modules within a PPS determine the length and the sequence of the synthesized peptide (Kleinkauf, H., von Döhren, H. (1990)
30 Eur. J. Biochem. 192:1-15). This is of fundamental importance because the structure of a product obtained after a replacement, insertion or deletion of PPS modules by genetic engineering can be predicted.

All known PPS modules share the feature that they are composed of at least three functional domains (Figure 1A). These three domains are (1) the adenylation domain, necessary for the recognition and adenylation of the substrate amino acid, and (2) the ACP domain, necessary for the covalent binding of the adenylation amino acid as thioester, and (3) the condensation domain, necessary for condensation of all PPS bound amino acids to the synthesized peptide (Stachelhaus et al. (1995) FEMS Microbiol. Lett. 125:3-14). Together, the adenylation domain and ACP domain are also described as activation domain (Figure 1A) because together they enable recognition and covalent binding of the substrate amino acid as a reactive thioester. A special group is formed by those activation domains which are also able to N-methylate their substrate amino acids after the covalent binding. With PPS having such activation domains, hence the peptide formed by the subsequent condensation also contains N-methylated amino acids. However, the number of presently known or cloned genes encoding for activation domains with N-methyltransferase activity is substantially lower than the number of activation domains without N-methyltransferase activity (more than 80 domains). Moreover, many of the domains with N-methyltransferase activity have a comparable substrate activity, e.g. for the amino acid in the modules of the actinomycin synthetase II from *Streptomyces chrysomallus* (Schauwecker et al. (1998) J. Bacteriol. 180:2468-2474), of the cyclosporine synthetase from *Tolypocladium niveum* (Weber et al. (1994) Cur. Genet. 26:120-125) and of the enniatin synthetase from *Fusarium scirpi* (Haese et al. (1993) Mol. Microbiol. 7:905-914).

The invention described hereinafter is important because it also allows the conversion of activation domains without N-methyltransferase activity into activation domains with N-methyltransferase activity without altering the original amino acid substrate specificity. Thus, for each specificity of a given PPS module, a corresponding module derivative with additional N-methyltransferase activity can be provided. These derivatives can then be used to construct novel or modified PPS by means of which the peptide synthesized by the PPS is N-methylated at the desired peptide bonds. This allows the synthesis of novel peptides with potentially new pharmacological properties. Many of the already known pharmacologically active peptides or peptide derivatives contain N-methylated amino acids, e.g. cyclosporine. In contrast to the invention, selective N-methylation of particular nitrogen atoms within the peptide bonds of polypeptides is only hard to accomplish or can even not be achieved by chemical methods.

The invention is based on the finding that all activation domains with N-methyltransferase activity harbor an additional domain which is localized between the adenylation and ACP domain (Figure 1B). This additional domain, designated N-methyltransferase domain hereinafter, mediates N-methylation of the bound substrate amino acid. The invention comprises methods for the conversion of activation domains without N-methyltransferase activity into activation domains with N-methyltransferase activity and the use thereof for reconstructing PPS for the synthesis of N-methylated amino acids and peptides. There are two basic approaches by which activation domains without N-methyltransferase activity of a PPS can be converted into activation domains with N-methyltransferase activity:

(1) Replacing a complete module or the complete activation domain of a PPS. This method is described in Example 2.

(2) Inserting a N-methyltransferase domain as a functional unit into an activation domain. For example, the N-methyltransferase domain can directly be inserted between the adenylation domain and ACP domain of the activation domain which is to be converted (Figure 2A). Two adjacent fusion sites can also be used for the insertion. In this case, the part between those fusion sites of the activation domain which is to be converted will be deleted and replaced by corresponding parts which will be inserted together with the N-methyltransferase domain (Figure 2B). This method is described in Example 3. If two fusion sites are used, the N-methyltransferase domain can also be inserted after the activation domain as an elongated unit with a tailing ACP domain (or parts thereof) leading to the replacement of the original ACP domain by the inserted ACP domain (or parts thereof) (Figure 2C and 2D). However, the substrate specificity of the converted activation domain is retained for each of the insertion approaches since the insertion does not alter the adenylation domain (recognition and adenylation of the substrate amino acid).

Suitable insertion sites for inserting a N-methyltransferase domain into an activation domain are determined by the transition between the adenylation domain and ACP domain. These result from the sequence comparison between activation domains with N-methyltransferase domain and activation domains without N-methyltransferase domain (Figure 3). The N-methyltransferase domains are located as insertions about 45 amino acids after (C-terminal) the adenylation domain consensus sequence QVKIRG(F/H/Y)RIE(L/I)GEIE, known as "core motif 5" (Turgay *et al.* (1992) *Mol. Microbiol.* 6:529-546),

The DNA sequence of the actinomycin synthetase II gene (*acmB*) has the "GenBank" data base entry AF047717. The DNA sequence of a 3849 bp *Bam*HI fragment, derived from the actinomycin synthetase III gene (*acmC*), is attached to the examples hereinafter.

Example 1 Introduction of a restriction site into the actinomycin synthetase II gene in order to enable the replacement of an activation domain.

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The actinomycin synthetase II (ACMS II) from *Streptomyces chrysomallus* possesses two modules without N-methyltransferase activity of which module 1 and 2 are activating threonine and valine, respectively. In order to be able to replace the activation domain of module 2, a *Eco*RV restriction site was introduced by mutagenesis into the ACMS II gene (*acmB*). This *Eco*RV restriction site and a *Cla*I restriction site which are already present in the gene allow to replace the region which encodes for the activation domain of module 2 by any given *Cla*I-*Eco*RV fragment. The replacement comprises numerous cloning steps which will be formally described first and thereafter in more detail.

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1. Formal summary of the cloning strategy

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Plasmid pACM5 was used to generate a *Eco*RV restriction site within the ACMS II gene (*acmB*) (Figure 4; Schauwecker et al. (1998) J. Bacteriol., 180:2468-2474). Plasmid pACM5 (Figure 4) harbors the gene *acmB* following a constitutive *Streptomyces* promotor (*melP*) and is a derivative of the *Streptomyces* plasmid pIJ702. An *Eco*RV restriction site was introduced by PCR mutagenesis and corresponding cloning steps into the gene *acmB* after the phosphopantetheine binding site

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encoding region (in module 2) at base pair (bp) position (pos.) 6251.

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5  acmB wildtype      (bp 6244-6262) :  5'- gtcgggacgtcttcgag
                                     V R D V F E
                                     (bp pos. 6251)

10 acmB mutagenized   (bp 6244-6262) :  5'- gtcgggatatcttcgag
                                     V R D I F E
                                     EcoRV
                                     (bp pos. 6251)

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2. Detailed description of the individual cloning steps

15 First, a 4923 bp *Pst*I-*Cla*I fragment, comprising the *mel* promoter and most of the 5'-located region of *acmB* (down to the *Cla*I restriction site at bp pos. 4519 in *acmB*), was isolated from pACM5 and cloned into *E. coli* plasmid pSP72 (A in Figure 5). Then, part of the adjacent 3'-region of *acmB*

20 (starting from the *Cla*I restriction site at bp pos. 4519) was amplified by PCR using the oligonucleotides *prim*-A and *prim*-B (PCR fragment 1 in Figure 5) and was inserted as 1737 bp *Cla*I-*Eco*RV fragment (B in Figure 5). Primer *prim*-B introduces an *Eco*RV restriction site corresponding to bp pos. 6251 in

25 *acmB*. The assembled fragments were then isolated as a complete *Pst*I-*Eco*RV fragment and cloned into pBlueScript (C in Figure 5). The assembled 5'-region of *acmB* can then be isolated as *Bam*HI-*Eco*RV fragment therefrom for subsequent cloning. The still missing 3'-region of *acmB* was amplified

30 using primer *prim*-C and *prim*-D (PCR fragment 2 in Figure 5) and was cloned as 2583 bp *Eco*RV-*Bam*HI fragment into pSP72 (D in Figure 5). The resultant plasmid was digested with *Bgl*II and *Eco*RV and the 5'-region of *acmB* (isolated as *Bam*HI-*Eco*RV fragment as described above) was inserted. This results in

35 plasmid pACM00-A (Figure 5) which harbors the completely

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A 2967 bp *ClaI-EcoRV* fragment of a 3849 bp *BamHI* fragment derived from the ACMS III gene (*acmC*, sequence is attached), which encodes for a valine activation domain with N-methyltransferase activity, was amplified by PCR using the oligonucleotides *prim-E* and *prim-F* (PCR fragment 4 in Figure 6). This *ClaI-EcoRV* fragment was cloned into the plasmid pACM00-A (from Example 1), whereby the *ClaI-EcoRV* fragment originally present in pACM00-A was replaced. The resulting plasmid was digested with *BamHI* and *HindIII* and the *Streptomyces* part from pSPIJ004 (Figure 4) was inserted as a 5130 bp *BglIII-HindIII* fragment. This generates the plasmid pACM00-B (Figure 7) which can be transformed and selected in both *E. coli* and *Streptomyces*.

Example 3 Conversion of an activation domain without N-methyltransferase activity into an activation domain with N-methyltransferase activity and introducing said converted activation domain into a PPS.

An additional N-methyltransferase domain was inserted into the valine activation domain of module 2 of ACMS II between the adenylation domain and the ACP domain. Thereby, the activation domain of ACMS II is provided with an additional N-methyltransferase activity. The inserted N-methyltransferase domain is derived from module 3 of the ACMS III. The replacement comprises numerous cloning steps which will be formally described first and thereafter in more detail.

1. Formal summary of the cloning strategy

First, two *Sna*BI restriction sites were introduced by PCR mutagenesis at bp pos. 5899 and bp pos. 5932 in gene *acmB* for the intended insertion of a N-methyltransferase domain. The region of 33 bp length between the two *Sna*BI restriction sites was then deleted and replaced by an 1263 bp *Eco*RV-*Eco*RV fragment encoding the above-mentioned N-methyltransferase domain of ACMS III. The ligation of the *Sna*BI ends with the *Eco*RV ends results in the formation of a DNA sequence which is no longer cleavable by both restriction enzymes. A new reading frame, encoding for a recombinant ACMS II, is obtained after inserting the *Eco*RV-*Eco*RV fragment for one of the possible two orientations.

12

R L V A Y V V A D G G T A P D G L R E A L
 ACMS II .. cgcctcgtcgcctacgtcgtcgcggaacggccccggacggtctgcgcgagggccctc ..
 (bp pos. 5899) (bp pos. 5932)

5

modified R L V A Y V R E A L
 ACMS II .. cgcctcgtcgcctacgtcgtcgcggaacggccccggactacgtacgcgagggccctc ..
 SnaBI SnaBI
 10 (bp pos. 5899) (bp pos. 5932)

N-methyltransferase domain I V A D L L T D
 15 of ACMS III gatATCGTCGCGGAC.....CTGCTCACCgATatc
 (1263 bp PCR-fragment) EcoRV EcoRV

recombinant R L V A Y I V A D L L T D V R E A L
 20 ACMS II .. cgcctcgtcgcctacATCGTCGCGGAC.....CTGCTCACCgATgtacgcgagggccctc ..
 (in plasmid pACM00-C) (bp pos. 5899) (bp pos. 7156)

The gene of the recombinant ACMS II (in plasmid pACM00-C,
 25 Figure 7) was transformed into *Streptomyces lividans* and the
 newly introduced N-methyltransferase activity of the
 recombinant PPS was verified as described in Example 4.

2. Detailed description of the individual cloning steps

30 In order to introduce the *SnaBI* restriction sites, the region
 of the gene *acmB* from bp pos. 4591 to 5899 as well as the
 region from bp pos. 5932 to 6251 were amplified by PCR using
 the oligonucleotides *prim-G* and *prim-H* (PCR fragment 1 in
 Figure 6) and *prim-I* and *prim-J* (PCR fragment 2 in Figure 6),
 35 respectively. Thereafter, the PCR fragment 2 was cloned as
 330 bp *HindIII*-*EcoRV* fragment into pBlueScript first and the
 PCR fragment 1 was then inserted as 1386 bp *ClaI*-*SnaBI*
 fragment. This results in a DNA fragment which encodes for
 the almost complete activation domain of module 2 of the ACMS
 40 II and in which a *SnaBI* restriction site was introduced (A in

liter of YEME. The purification of PPS up to a stage necessary for enzymatic analysis is essentially based on a protocol previously described in detail (Schauwecker *et al.* (1998) J. Bacteriol. 180:2468-2474) and is therefore described only schematically:

Proteins were released from cells by mechanic cell disruption (French press). Simultaneously released genomic DNA was digested with DNaseI to obtain a fluid suspension. Cell fragments were removed by centrifugation and proteins were then precipitated by addition of ammonium sulfate up to a final concentration of 55%. Precipitated proteins were size-fractionated by exclusion chromatography (column matrix: Ultrogel-AcA-34 from Biosepra). Protein fractions with protein having a size larger than 200 kDa were pooled and further purified on an anion exchanger (column matrix: Q-Sepharose FF from Pharmacia). Proteins bound on the anion exchanger were release from the anion exchanger by continuously adding NaCl. The PPS which were constructed according to Examples 2 and 3 eluted in a range between 150 to 250 mM NaCl. PPS partially purified according to this protocol can then be further analyzed, for example according to the protocols as given below:

Example protocol as to how to verify the specific recognition and binding of amino acids by a PPS *in vitro*:

Mix 100 μ l of a partially purified PPS with 3 μ l of ^{14}C -labelled substrate amino acid (100 $\mu\text{Ci/ml}$), 2 μ l MgCl_2 (1 M) and 15 μ l ATP (0.1 M) and incubate for 30 minutes at 30 $^\circ\text{C}$. Precipitate the PPS by adding 2 ml of 7% trichloroacetic acid (TCA), wash with 10 ml 5% TCA and quantify the amount of substrate amino acid bound to the enzyme by measuring the radioactivity.

Example protocol as to how to verify the N-methylation of substrate amino acids catalyzed by a PPS *in vitro*:

- 5 To verify the N-methylation activity, incubate the PPS with ^{14}C -labelled substrate amino as described above but complement the incubation mix by adding 3 μl of 0.1 M S-adenosyl methionine (SAM) as a donor of the methyl group which is to be transferred to the amino acid. After TCA
- 10 precipitation, wash the PPS with 4 ml of 5% TCA (two portions), then wash with 2 ml ethanol and dry at 37 °C. Add 300 μl performic acid and incubate for 6 hours at 20 °C to release the substrate amino acid bound as thioester. Then vacuum dry the mixture. Dissolve the amino acid by adding 40
- 15 μl formic acid and verify the conversion into the N-methylated form, e.g. by chromatographic methods. For example, the conversion of valine into N-methyl valine can be shown as follows: Chromatograph 20 μl of the (^{14}C -labelled) amino acid released from the PPS in parallel to 5 μl of the
- 20 corresponding references (0.1 M valine and 0.5 M N-methyl valine) on a silica 60 thin-layer chromatography plate (Merck) using the solvent system n-butanol : acetic acid : water (volume 80:20:20). Visualize the amino acids by a ninhydrin reaction and a autoradiogram for the ^{14}C -labelled
- 25 amino acid.

Example protocol as to how to verify the formation of peptides catalyzed by a PPS *in vitro*:

- 30 In general, a peptide can simply be analyzed by acidic hydrolysis followed by the determination of the individual amino acid components. This applies especially to peptides which are formed by PPS since the amino acid sequence of the

synthesized peptide is already known from the module arrangement. Because of the use of ^{14}C -labelled amino acids, the analysis of the *in vitro* formed peptide can be performed as follows: Incubate 100 μl of partially purified PPS with
5 each of the PPS substrate amino acids (2 mM each), SAM (2 mM), ATP (10 mM) and MgCl_2 (20 mM) in a total volume of 150 μl for 25 minutes at 30 $^\circ\text{C}$. If necessary, the mixture may contain further enzymes which are co-acting with the PPS which is intended to be analyzed (Pfennig et al. (1999) JBC
10 274:12508:12515). Prepare more than one incubation mixture in parallel, in which the number of the incubation mixtures is dependent on the number of modules within the PPS and use the ^{14}C -labelled amino acid which corresponds to the module in each of the incubation mixtures. Precipitate the PPS in each
15 of the incubation mixtures with TCA as described above, cleave off the synthesized peptide with performic acid, dry and dissolve the formed peptide in ethanol / water (volume 1:1) and verify the peptide by chromatographic methods. For example, to verify the threonyl-N-methyl-valine peptide
20 linkage by the PPS constructed according to Examples 2 and 3 one can proceed as follows: Chromatograph 20 μl of the peptide released from the PPS of each incubation mixture (one with ^{14}C -labelled threonine and one with ^{14}C -labelled valine) on a silica 60 thin-layer chromatography plate (Merck) using
25 the solvent system n-butanol / acetic acid / water (volume 80:20:20). Isolate all products formed in both incubation mixtures having an identical R_f value by extraction using ethanol / water (volume 1:1), vacuum dry and release the amino acids from the peptides by acidic hydrolysis (6 N HCl,
30 110 $^\circ\text{C}$, 20h). The identification of the released ^{14}C -labelled amino acids is again performed by chromatography on thin-layer chromatography plates using the same solvent system. This allows to identify the components threonine and N-

methy1-valine in the formed peptide. Furthermore, a peptide reference can directly be compared with the enzymatically formed and ^{14}C -labelled peptide, e.g. by HPLC using a column designed for peptide separation like the SuperPac Pep-5
 5 column from Pharmacia, if synthesis of the reference peptide by chemical means is possible.

Tables and Figures

Table 1

Starting plasmids used for realizing the Examples

plasmid	origin or literature quotation	selection	description
pSP72	Promega	Amp	commercial cloning vector for <i>E. coli</i>
pBlue-Script	Stratagene	Amp	commercial cloning vector for <i>E. coli</i>
pIJ702	Katz <i>et al.</i> (1983) J. Gen. Microbiol. 129 : 2703-2714	Tsr	Commonly used cloning vector for <i>Streptomyces</i> . It harbours the melanin (<i>mel</i>) genes <i>melC1</i> and <i>melC2</i> under control of their promotor (<i>mel P</i>).
pSPIJ004	own development	Amp Tsr	The plasmid is a combination of pSP72 and pIJ702 and is replicable both in <i>E. coli</i> and in <i>Streptomyces</i> . For this purpose, the <i>PstI</i> - <i>BglIII</i> fragment from pIJ702 was cloned into the polylinker of pSP72.
pACM5	Schauwecker <i>et al.</i> (1998) J. Bacteriol. 180 : 2468-2474	Tsr	The plasmid is a pIJ702 derivative and harbours the actinomycin synthetase II gene (<i>acmB</i>) under control of the <i>mel</i> -promotor.

abbreviations: Tsr = thiostreptone, Amp = ampicillin

Table 2

PCR oligonucleotides used in the Examples

oligonucleotide	DNA sequence and restriction sites
prim - A	5'- gccggaattcgtatcgatgtcctcaccgccgaggaga EcoRI ClaI
prim - B	5'- tgcggaattcgaagatatcccgacggagaaaccgat EcoRI EcoRV
prim - C	5'- tctccgtccgggatattctcgagcagcgcacg EcoRV
prim - D	5'- atggcctgagttgctggatcctggcgatcccga BamHI
prim - E	5'- ctgagccgcacatcgatgtcctca ClaI
prim - F	5'- cgctcgaagatatcgcgaggccca EcoRV
prim - G	5'- gcaggaattcagccgtatcgatgtcctca EcoRI ClaI
prim - H	5'- ttccggaattcgcgactacgtaggcgacga EcoRI SnaBI
prim - I	5'- cggccaagctttacgtacgcgaggccctccggcggcgct HindIII SnaBI
prim - J	5'- tgcggaattcgaagatatcccgacggagaaaccgat EcoRI EcoRV

	nucleotide sequence	numbering of base pairs
5	GGATCCACCT GCTCGACACC GCCACCGCCC AACC CGAGCA GCCCCTCAGC CGCATCGACG	0000000060
	TCCTCACCCC GGAGGAGAGG AACCGCACGA TCGTCGAGGT CAACCGGACC GAACTGCCGC	0000000120
	TGCCCCGACG CTCGTTGGCG GAGCTGTTTCG AACAAACAGT GACCCCTCACA CCGACGCCC	0000000180
	CCGCCCTGGT CAGCGACGGC GCCACGCTCA GCTACTCGA GCTCAACACG CGCGCCAACC	0000000240
10	ACCTCGCCCA CCAGCTCACC ACCCGGGGCA TCCGCCCCG CGACGCCGTC GCCGTCTCTC	0000000300
	TCCAACGCTC CCCCACACC GTCACCACCG TCCTCGCCCT CGCCAAGACC GGC GCGACCT	0000000360
	ACATCCCCCT CGACAGCCGC TACCCCGCCG ACCGCTACCG CCTCGTCTC GACGAGACCC	0000000420
	GCACCAAACCT CCTCATCACC GACCACACCA CCGACCTCGA CACCACCACA ACCCAGTTCA	0000000480
	ACCCCGCCGA CACCCCCCAC GACGGCGAAG ACCCCGGCAA CCCGAACCAC ACCACCCACC	0000000540
15	CCGACGACGC CGCCTACATC ATGTACACCA GCGGCTCCAC CGGCGCCCC AAGGGCGTCA	0000000600
	TCGCCACCCA CCGCAACATC ACCGCCCTCG CCCTCGACCC CCGCTTCGAC CCCACCGCCC	0000000660
	ACCGCCGCGT CTCTCTCCAC TCCCCACCG CCTTCGACGC CTCACCTAC GAGATCTGGG	0000000720
	TCCCCCTCCT CAACGGCAAC ACCGTCGTCC TCGCCCCCAC CGGCGACCTC GACGTCCACA	0000000780
	CCTACCACCG CGTCATCACC GACCAGCAGA TCACCGCCCT CTGGCTGACC AGCTGGGTCT	0000000840
20	TCAACCTCCT CACCGAGCAG AGCCCGGAGA CCTTCACCCG GGTCCGGCAG ATCTGGACCG	0000000900
	GCGGCGAGGC CGTCTCCGGC GCCACCGTCA CCGGCTTCA GCAGGCATGC CCCGACACCA	0000000960
	CCGTGGTTCGA CGGTACGGC CCCACCGAGA CCACCACCTT CGCCACCCAC CACCCCGTCC	0000001020
	CCACCCCTTA CACCGGCTCC GCCGTCTGTC CCATCGGCCG CCCCATGGCC ACCATGCACA	0000001080
	CCTACGTGCT CGACGACAGC CTCAGCCCG TCGCCCCCG CGTCAACGGC GAGCTCTACC	0000001140
25	TCGCTGGCAG CGGCCTCGCC CGCGGCTACC TGGACCGCCC CGCCCTCACC GCCGAACGCT	0000001200
	TCGTGCGCAA CCCGTACGCC GCACCCGGAG AACGCATGTA CCGCACCGGC GACCTGGCAC	0000001260
	GCTGGAACCC CGACGACCAC CTCGAGTACG CCGGCGCGC CGACCACCAG GTCAAGGTCC	0000001320
	GCGGCTTCCG CATCGAACCC GCGGAGATCG AGAACGTCTT CACCAGCCAT CCCGCCGTCG	0000001380
	CCCAGGCCCG CGTCCACCTC AACCGGGACC AGCCCGGCAA CCCC CGGCTC GTCCGCTACG	0000001440
30	TCGTGCGGGA CACCTCGGCG CCGAGCAGCG ATGTGGACCA GCAGCACCAG ATCGGCGAGT	0000001500
	GGCAGGACCT CTACGACTCC CTCTACGCGG CCCCCACGGC CGAGTTTCGGC GAGGACTTCT	0000001560
	CCGGCTGGAA CAGCAGCTAC GACGGCCGGC CGATCCCCCT CGACCAGATG CGGGAGTGGC	0000001620
	GCGACGCCAC CGTGGAAACG ATCCGCGGCC TCAACCCGCG CCGGGTGCTG GAGATCGGCG	0000001680
	TCGGCACGGG CCTGTCTGTC GCGAAGCTGG CCCCAGAGTG CGAGGAGTAC TGGGGCACGG	0000001740
35	ACCTCTCGCC CACCGTGATC GAGGCCTCT CCCGGCACGT CGACGCCGAC CCGGAGCTGG	0000001800
	CCCGCGGGGT CACCTTGGCG GCCGTGCGG CGCACGAGCA CGAGGGGCTG CCCGTGCGCC	0000001860
	ACTTCGACAC CGTCGTGCTC AACTCCGTGG TCCAGTACTT CCCGAACGCC GACTACCTCG	0000001920
	CCCAGGTCAT CGAGCAGGCG CTGCGGCTGC TGGCCCCCG CGGCGCGCTG TTCATCGGCG	0000001980
	ACATCCGCAA CCCGCGGCTG CTGCGCACCT TCACCACCGC CGTCCAGACC GCCCGCGCGG	0000002040
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	AGGAACCTCT GGTTCGACCG GAGTACTTCA CCGCGCTCAC CCACCGCTC CCGGACCTCG	0000002160
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	ACGACACCAC GCTCCACAAG GCCGGAATCA CCGCGCTCCC GCTGTCCGAG GCGCCGCTCC	0000002280
	TGGCCTGGCC GCAGGACGCC GAGGCACTCG CCCGGCACCT GGCCGAGGCC CGGCCGGAGC	0000002340
45	GGCTGCGCGT CACCGGCGCG CCCAACTCCC GGATAGCCGC CGACCTCGCG GCCCAGCAGC	0000002400
	CCCTGGAGTC CGGCACCGCC CCGGCCGGGC CCCCAGCCGG GCCCTACGCC ACGGAGCAGC	0000002460
	CGGACCTCGA GCACTCCAC CGCCTCGGGG AGGACCACGG GTACTGGACG GCCGTCACTT	0000002520
	GGTCCGCCCA CCGCCCCGAC ACCGTGACCT TCACCTTCGT CCGGCGCGGC CTGCTCGACG	0000002580
	GCGCCGTCCC GGTCGGTACG TACGCCCCGG CGGCCCGCGG CGACCCGGCG ACGCCGCTCA	0000002640

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	ACCGCCTGCC	GCTCACCGCC	AACGGCAAGC	TCGACCGGGC	CGCCTCCCG	GCACTCGACC	000002820
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5	TGTTGCGGGA	GGTGCTCGGC	CGGCCGCTCG	TCGGTGTGGA	CCAGGACTTC	TTCGACCTCG	000002940
	GCGGGCACTC	GCTGCTCGCC	ACCCGCTGTA	TCGCCCGGCT	GCGCGCCGCC	TTCGGCGTGG	000003000
	AACTGGGCCT	GCGCAGCCTC	TTCGAGGCGC	CGACGCCGGG	CGGGATCGCC	GCCCGGCTGG	000003060
	ACCTCGACGA	CCCGGACGGC	TCCTACGAGG	TGGTGCTGCC	GCTGCGCGCC	CAGGGCAGCA	000003120
	GGCCGCCGCT	GTTCTGCATC	CACCCCGGTG	GCGGCATCAG	CTGGTCGTAC	AGCGCGCTGA	000003180
10	TCAAGCACCT	CGGCCCGGAG	TACCCGCTGT	ACGGCATCCA	GGCGCGCAGC	CTGGCCCGCC	000003240
	CGGAGCCGCG	GCCGGAGAGC	ATCGAGGAGA	TGGCGGTGGA	CTACGCCGAC	CAGATCCAGG	000003300
	GCGTGCAGCC	GCACGGCCCC	TACCACCTGG	CCGGCTGGTC	GTTCCGGCGGG	CTGTGCGCCC	000003360
	ATGCCCTGGC	CGCGGAGTTC	CAGCGGCGCG	GCGAGCCGGT	GGCGCTGGTC	GCGGTGCTCG	000003420
	ATGTGATCCC	GAAGTGGCAG	GGGCTCACCC	ACGACGACGT	CCCGGCCCCC	GACGACCGGG	000003480
15	TGATGCTGCT	GTACCACGTC	GGCCTGGTCG	ACGACGGCAG	CCACCGCAAC	GACCGCGAAG	000003540
	AGCTGACCTT	CGCCAGGGCC	CGCGAGATCC	TGCGCCGCCA	GGGCAGTGTG	CTCGCCAACC	000003600
	TGGAGGAGGA	CCGGCTCACC	ACGATCACCG	AGATCTCGGC	CAACAACACC	CATCTGACCC	000003660
	TCGACTACCA	GCCCCGCCCC	ATCGACGGCG	ACCTGCTGCT	GATCGCGGCC	TCGGAACAGC	000003720
	AGGACCCGCC	GGTCAACGCC	GATGCCTGGC	GGCCGTACGT	CTGCGGCGCG	GTCGAGGCCC	000003780
20	ACGTGGTGCC	CGGCGAGCAC	GGCTCCATGC	TGACCCGGCC	CGGCACCCTG	GCCGAGATCG	000003840
	GCCGGATCC						000003849

Figure 1: shows the schematic modular set-up of PPS and the subdivision in functional domains.

5 Figure 2: shows the modification of activation domains by insertion of a N-methyltransferase domain.

Figure 3: shows the sequence comparison of selected activation domains in the transition regions towards the N-methyltransferase domains.

10

Figure 4: shows the starting plasmids used in the Examples.

Figure 5: shows the introduction of an *EcoRV* restriction site into *acmB*.

15

Figure 6: shows the cloning of *ClaI-EcoRV* cassettes for the construction of recombinant *acmB* genes.

20 Figure 7: shows plasmids for the expression of recombinant PPS genes.

Claims:

1. Method for the manufacture of a recombinant DNA encoding for a polypeptide synthetase (PPS) activation domain
5 with N-methyltransferase activity, wherein a first DNA fragment encoding for a domain with N-methyltransferase activity is cloned into a second DNA fragment encoding for a PPS activation domain without N-methyltransferase activity, and wherein the first and the second DNA fragment form a
10 continuous reading frame.

2. Method for the manufacture of a recombinant DNA encoding for a PPS with N-methyltransferase activity, wherein the first DNA fragment according to claim 1 is cloned into a
15 second DNA fragment encoding for a PPS with an activation domain without N-methyltransferase activity at the DNA region encoding for the activation domain without N-methyltransferase activity, and wherein both DNA fragments form a continuous reading frame.

20

3. Method for the manufacture of a recombinant DNA encoding for a PPS with N-methyltransferase activity, wherein a DNA fragment of a PPS gene encoding for an activation domain without N-methyltransferase activity is replaced by
25 the recombinant DNA obtained according to claim 1 or by a DNA fragment, which is encoding for a natural activation domain with N-methyltransferase activity, and wherein a continuous reading frame is obtained.

30

4. Method according to any of claims 1-3, wherein the DNA fragment encoding for a domain with N-methyltransferase activity is cloned between the DNA regions encoding for the adenylation domain and for the ACP-domain of the PPS

activation domain without N-methyltransferase activity by means of a single fusion site.

5 5. Method according to any of claims 1-3, wherein the (DNA fragment encoding for a domain with N-methyltransferase activity is cloned by means of two fusion sites.

10 6. Method according to any of claims 4 or 5, wherein the DNA fragment encoding for a domain with N-methyltransferase activity is additionally encoding for an ACP domain, an activation domain or a condensation domain.

15 7. DNA, obtainable according to the method of any of claims 1-6.

 8. Cell, containing at least one DNA according to claim 7.

20 9. Method for the manufacture of a PPS with N-methyltransferase activity, wherein the DNA obtained according to the method of any of claims 2-6 encoding for a PPS with N-methyltransferase activity is expressed.

25 10. Method according to claim 9, wherein the PPS is encoded on a plasmid and the expression is carried out in a microorganism.

30 11. PPS with N-methyltransferase activity, obtainable according to the method of any of claims 9-10.

 12. Use of the PPS according to claim 11 for the catalytic influence on an educt compound or a mixture thereof.

13. Product compound, obtainable by the catalytic influence of the PPS according to the use of claim 12 on an educt compound or a mixture thereof.

5

14. Use of the product compound according to claim 13, to test for its pharmacological effectiveness.

15. Use of the DNA according to claim 7 for the
10 manufacture of recombinant PKS genes or recombinant gene segments thereof.

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES
PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro



(43) Internationales Veröffentlichungsdatum
21. Dezember 2000 (21.12.2000)

PCT

(10) Internationale Veröffentlichungsnummer
WO 00/77220 A3

- (51) Internationale Patentklassifikation⁷: C12N 15/52, (71) Anmelder und
15/54, 15/62, 9/10, C12P 13/04, 21/02 (72) Erfinder: KELLER, Ullrich [DE/DE]; Selbitzerstr. 16 c,
D-14089 Berlin (DE).
- (21) Internationales Aktenzeichen: PCT/DE00/01950 (72) Erfinder; und
- (22) Internationales Anmeldedatum: 15. Juni 2000 (15.06.2000) (75) Erfinder/Anmelder (nur für US): SCHAUWECKER,
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- (25) Einreichungssprache: Deutsch (74) Anwalt: NOBBE, Matthias; Viering, Jentschura & Part-
ner, Essener Strasse 5, D-46047 Oberhausen (DE).
- (26) Veröffentlichungssprache: Deutsch
- (30) Angaben zur Priorität: 199 28 313.3 16. Juni 1999 (16.06.1999) DE (81) Bestimmungsstaaten (national): AE, AG, AL, AM, AT,
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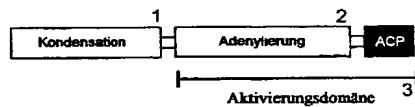
[Fortsetzung auf der nächsten Seite]

(54) Title: METHOD OF MODIFYING PEPTIDE SYNTHETASES SUCH THAT THEY CAN N-METHYLATE THEIR SUB-
STRATE AMINO ACIDS

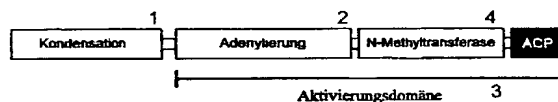
(54) Bezeichnung: VERFAHREN ZUR VERÄNDERUNG VON PEPTIDSYNTHETASEN IN DER WEISE, DASS SIE IHRE
SUBSTRATAMINOSÄUREN N-METHYLIEREN KÖNNEN

Abbildung 1: Modul einer PPS und Unterteilung in funktionelle Domänen
MODULE OF A PPS AND SUBDIVISION INTO FUNCTIONAL DOMAINS

A: Minimal-Modul einer PPS



B: Modul mit N-Methyltransferase-Domäne



A: MINIMAL MODULE OF A PPS

1...CONDENSATION

2...ADENYLATION

3...ACTIVATION DOMAIN

B: MODULE WITH N-METHYL TRANSFERASE DOMAIN

4...N-METHYL TRANSFERASE

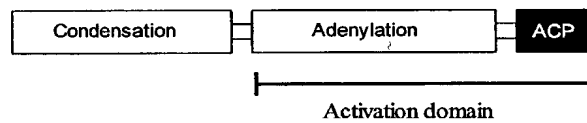
(57) Abstract: The invention relates to a method of modifying peptide synthetases in such a manner that they can N-methylate their substrate amino acids. PPS are enzymes that synthesize peptides in a non-ribosomal manner. PPS have a modular set-up. Each module has an activation domain which recognizes and covalently binds to the respective substrate amino acid. The peptide synthesis catalyzed by PPS proceeds by the condensation of the covalently bound substrate amino acids. A minor number of the known activation domains is capable of also N-methylating the bound substrate amino acids. The inventive method allows conversion of the activation domains without N-methyl transferase activity to activation domains with N-methyl transferase activity while maintaining the original substrate specificity.

[Fortsetzung auf der nächsten Seite]

WO 00/77220 A3

Figure 1: Module of a PPS and subdivision into functional domains

A: Minimal module of a PPS



B: Module with N-methyltransferase domain

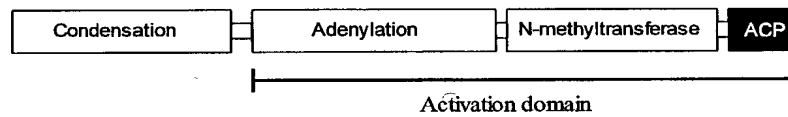
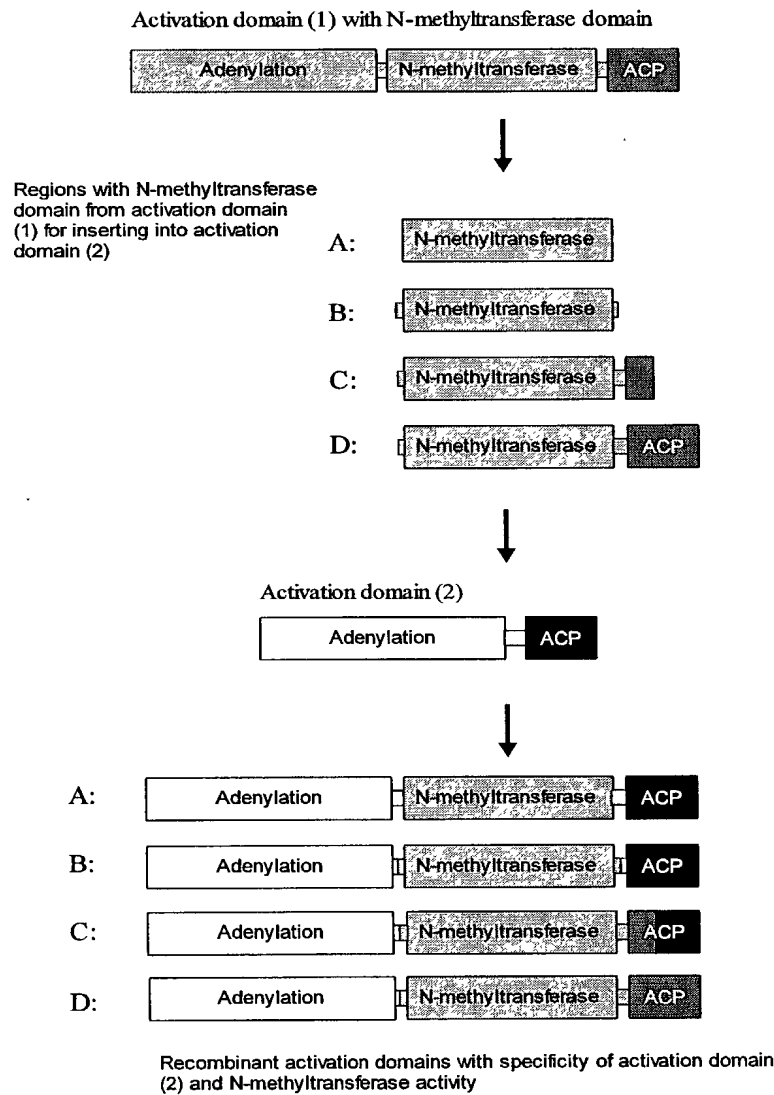


Figure 2: Conversion of activation domains by inserting a N-methyltransferase domain



3b/7

CYS A	Cyclosporine Synthetase from Tolypocladium niveum (Z28382)
GRS A,B	Gramicidin S Synthetase I,II from Bacillus brevis (P14687/P14688)
ACM B,C	Actinomycin Synthetase II,III from Streptomyces chrysomallus (AF047717 and attached sequence)
BAC A,B,C	Bacitracin Synthetase A,B,C from Bacillus licheniformis (AF007865)
TYC A,B,C	Tyrocidin Synthetase I,II,III from Bacillus brevis (AF004835)
LIC A,B,C	Lichenysin Synthetase A,B,C from Bacillus licheniformis (U95370)
ESYN	Enniatin Synthetase from Fusarium scirpi (Z18755: Update year 2000)
SNB C,D	Pristinamycin I Synthetase C,D from Streptomyces pristinaespiralis (Q54959,X98690)
SRF 1,2,3	Surfactin Synthetase 1,2,3 from Bacillus subtilis (P27206,Q04747,Q08787)
PPS 1,2,3,4	Fengycin Synthetase 1,2,3,4 from Bacillus subtilis (Z34883)

The index following the name designates the number of the activation domain within the corresponding PPS (started from the N-terminus). The data base numbers of the sequences in "GenBank" or "SwissProt" are given in parenthesis.

4/7

Figure 4: Starting plasmids for the construction according to the Examples

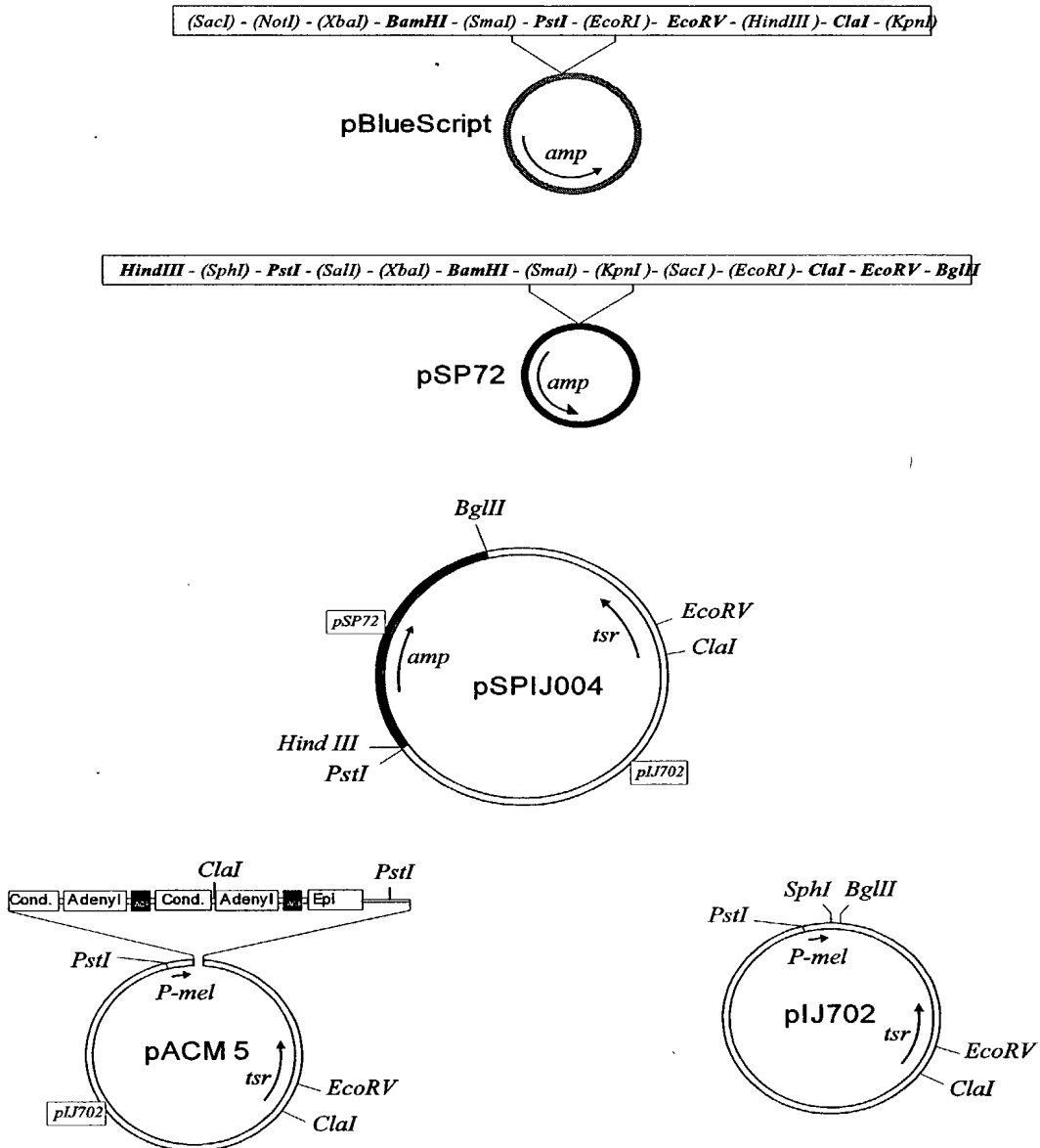


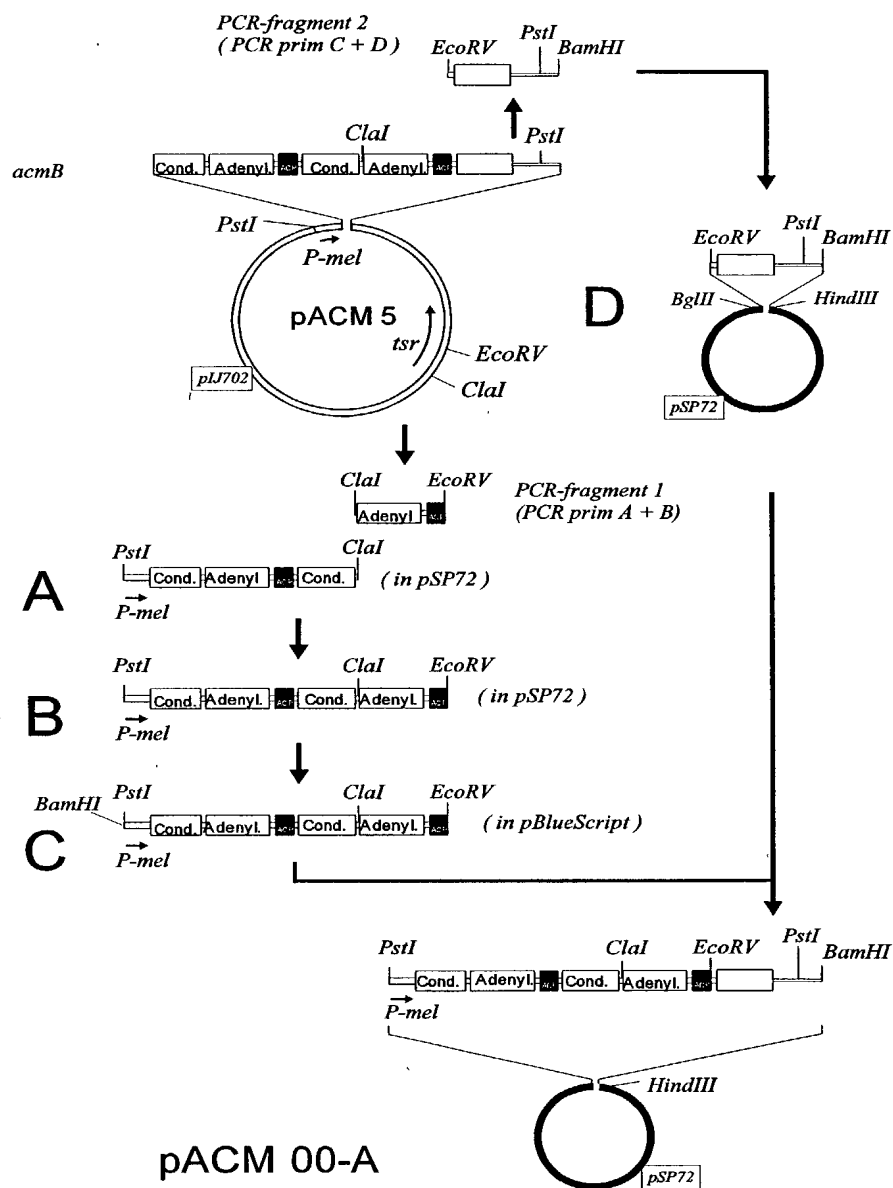
Figure 5: Introduction of an *EcoRV* restriction site into *acmB*

Figure 6: Cloning of *ClaI*-*EcoRV* cassettes for the construction of recombinant *acmB* genes

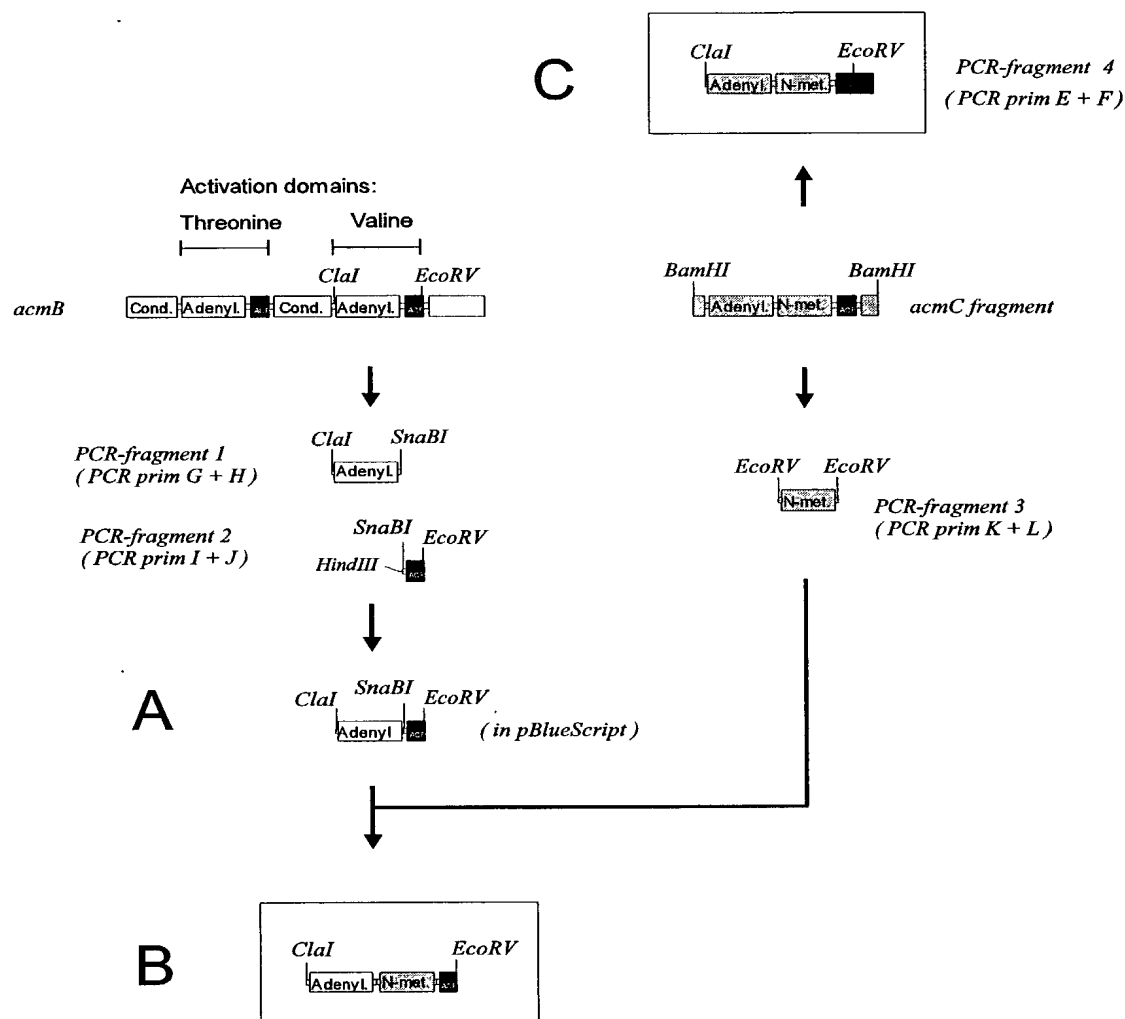
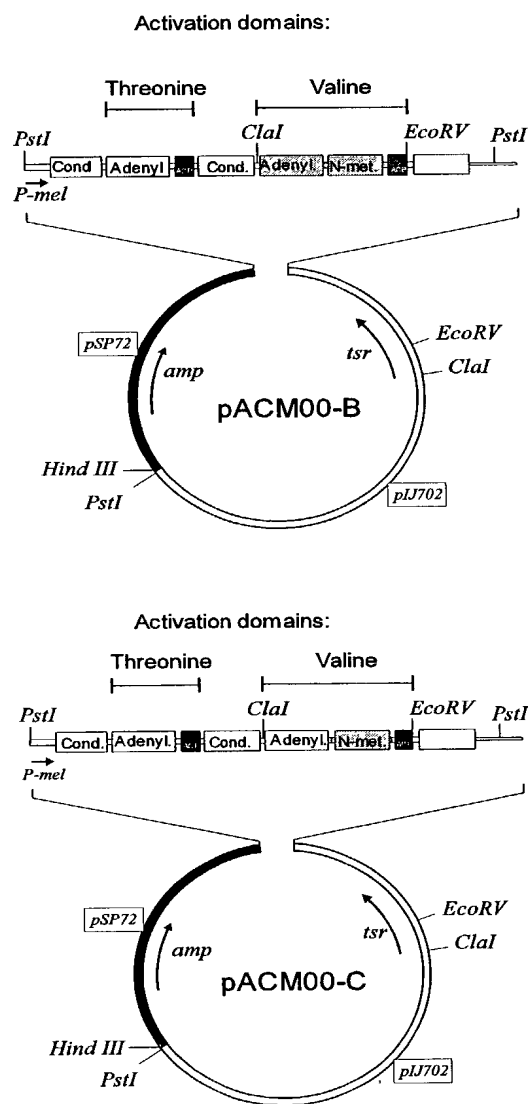


Figure 7: Plasmids for expression of the recombinant PPS genes



1
SEQUENZPROTOKOLL

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koennen

<130> KeActinoPat1

<140> PCT/DE00/01950

<141> 2000-06-15

<150> DE-19928313.3-41

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Practitioner's Docket No. **100806.01-US1**

PATENT

COMBINED DECLARATION AND POWER OF ATTORNEY
(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL,
CONTINUATION, OR C-I-P)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is for an original application.

INVENTORSHIP IDENTIFICATION

My residence, post office address and citizenship are as stated below, next to my name. I believe that I am an original, first and joint inventor of the subject matter that is claimed, and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

METHOD OF MODIFYING PEPTIDE SYNTHETASES SUCH THAT THEY CAN N-METHYLATE THEIR SUBSTRATES.

SPECIFICATION IDENTIFICATION

The specification was described and claimed in PCT International Application No. PCT/DE00/01950 filed on June 15, 2000.

ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information, which is material to patentability as defined in 37, Code of Federal Regulations, Section 1.56.

PRIORITY CLAIM (35 U.S.C. Section 119(a)-(d))

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America

filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Such applications have been filed as follows.

**PRIOR PCT APPLICATION(S) FILED WITHIN 12 MONTHS
(6 MONTHS FOR DESIGN) PRIOR TO THIS APPLICATION
AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. SECTION 119(a)-(d)**

INDICATE IF PCT	APPLICATION NUMBER	DATE OF FILING DAY, MONTH, YEAR	PRIORITY CLAIMED UNDER 35 U.S.C. SECTION 119
PCT	PCT/DE00/01950	15 June 2000	Yes

**ALL FOREIGN APPLICATION(S), IF ANY, FILED MORE THAN 12 MONTHS
(6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION**

German Application No. 199 23 313.3 filed 16 June 1999

POWER OF ATTORNEY

I hereby appoint the following practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

APPOINTED PRACTITIONER(S)	REGISTRATION NUMBER(S)
Robert D. Fish	<u>33,880</u>
David J. Zoetewey	<u>45,258</u>
Sandra Poteat Thompson	<u>46,264</u>
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I hereby appoint the practitioner(s) associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

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Practitioner's Docket No.

PATENT

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

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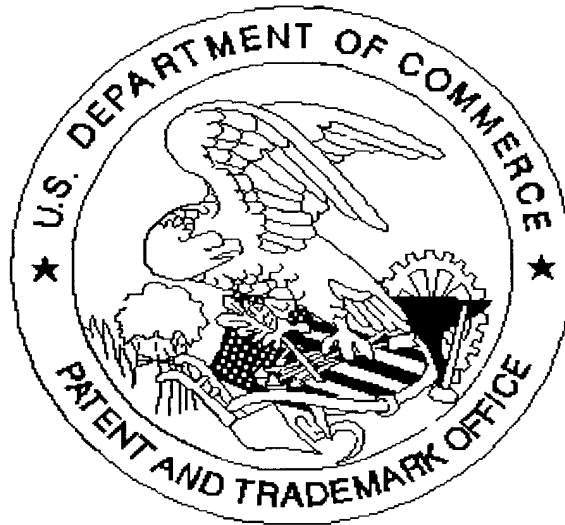
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